Instrumentation. **Chromatography: Liquid:** Partition Chromatography (Liquid–Liquid). **III/Chiral Separations:** Countercurrent Chromatography; Liquid Chromatography. **Foam Countercurrent Chromatography. Ion Analysis:** Liquid Chromatography; High-Speed Countercurrent Chromatography. **Liquid Chromatography. Medicinal Herb Compounds: High-Speed Countercurrent Chromatography. Natural Products:** High-Speed Countercurrent Chromatography; Liquid Chromatography. **Peptides and Proteins:** Liquid Chromatography. **Proteins:** High-Speed Countercurrent Chromatography.

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Derivatization

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Introduction

Derivatization involves changing in some way the basic chemical or physical structure of a compound, usually to a single product, which may be more useful for the analysis of the original analyte in liquid chromatography (LC). Derivatization can be used for analytical or preparative scale LC. In the analytical mode, it can be used to improve the identification and quantitation of the analyte of interest. It may also be used to improve throughput and recovery in preparative scale LC purifications of large amounts of material. Changes in the basic structure of the analyte can also lead to improved peak shape, elution times, peak symmetry, efficiency, plate count, and other indicators of chromatographic performance. That is, elution times and retention factors, as well as resolution, separation factors, reduced plate heights, and other LC parameters of performance, can all be varied and improved by suitable, selective derivatization of the starting analyte.

The most general type of derivatization involves modifying the chemical structure of the starting compound by tagging or adding another reagent to it via a suitable functional group alteration (**Figure 1**). Thus, most simple derivatizations involve a derivatizing reagent, the substrate or analyte of interest, the desired derivative of the analyte, remaining excess reagent, and undesirable by-products coming from the excess derivatizing reagent reacting with solvent, water or thermally degrading (Figure 1). Ideally, only the desired derivative would remain at the end of the reaction period, without any remaining starting analyte, derivatizing reagent or by-products. However, this idealized situation is rarely observed and it is often necessary to separate prior to or during the LC analysis the desired derivative from all other possible compounds coming from the derivatization reaction and/or sample components and their possible derivatization products.

Though most derivatizations usually occur in a homogeneous solution between the analyte of interest and the reagent itself, it is possible to perform derivatizations on the analyte in solution with an immobilized or solid-phase reagent. **Figure 2** illustrates a typical immobilized or solid-phase reagent that has been described in the literature for use with LC. It is also feasible to first immobilize the analyte on a solid support, such as silica gel, Immobilon™ membrane, poly(styrene-divinylbenzene), C_{18} packing material, and others, and then perform the derivatization reaction on the now-immobilized analyte. Once the reaction is completed, the excess reagent is simply washed from the solid support still containing the derivative. The desired derivative is then eluted with a stronger solvent from the solid support, often in a disposable plastic tube (solid-phase extraction cartridge or Sep-Pak™), without any residual, unreacted starting

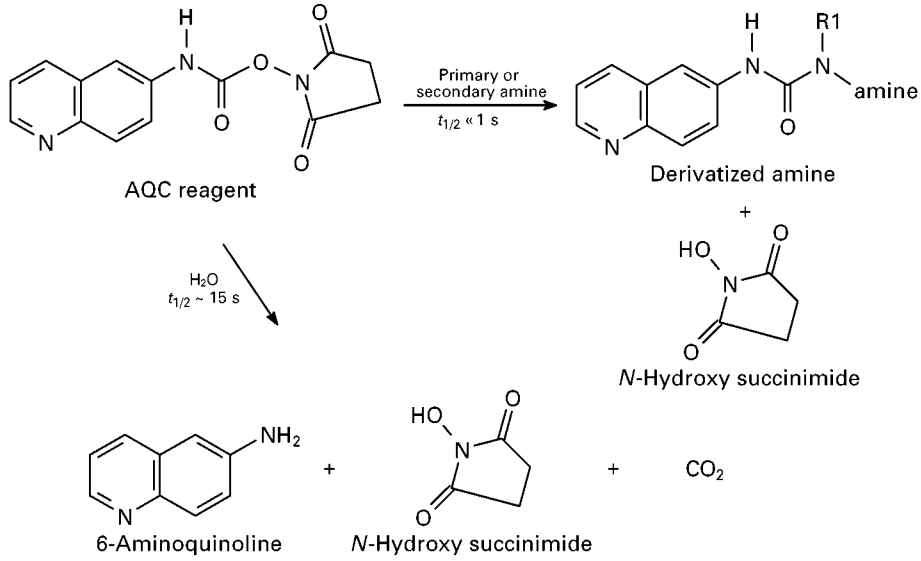

Figure 1 Chemical derivatization of an analyte using 6-aminoquinoyl-*N*-hydroxysuccinimidyl carbamate (6-AQC) reagent. This is a homogeneous reaction that occurs in solution. The 6-AQC degrades with water to form 6-aminoquinoline and *N*-hydroxysuccinimide, with the release of carbon dioxide.

analyte or by-products of the reagent, in a state suitable for direct LC injection.

Other approaches to derivatization involve the use of photochemical reactions, usually performed online after the separation occurs, which convert the starting analyte into one or more derivatives with improved detection properties (ultraviolet (UV), fluorescent (FL), electrochemical (EC), etc). This does not introduce excess derivatizing reagent, reagent by-products or hydrolysis products, since the reagent itself is light rather than a chemical. Such approaches have become popular in LC applications. It is also possible to utilize electrochemistry to perform derivatizations in LC, as well as microwave digestion, immobilized enzyme reactors, pH alteration of the mobile phase after the separation, etc. **Table 1** summarizes the most commonly utilized derivatization techniques described in LC other than simple, chemical reactions.

General Approaches to Derivatization in Liquid Chromatography

Chemical derivatization in LC requires the optimization of several reaction or separation parameters. These include temperature, pH, solvent, time, ratio of

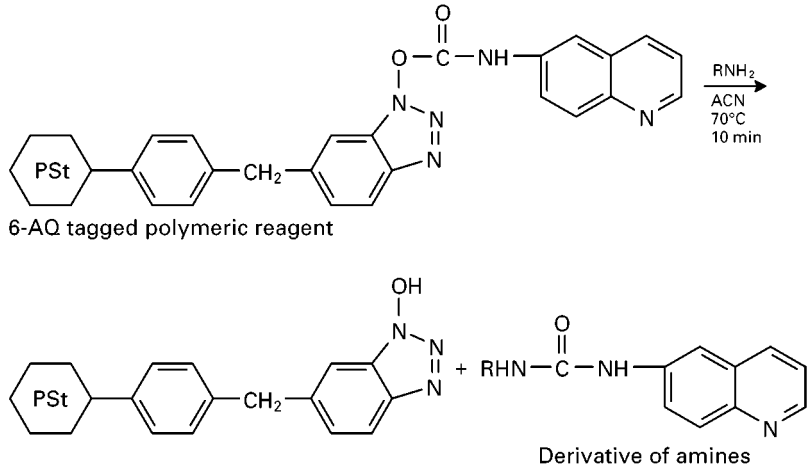

Figure 2 Typical immobilized or solid-phase reagent. The 6-AQ-tagged polymeric reagent reacts with amines (70°C, 10 min), producing a derivative free in solution, now 6-AQ derived. (6-AQ, 6-aminoquinoline; PSt, polystyrene.)

Table 1 Summary of common derivatization approaches, other than chemical reactions, used in LC

1. Photochemical conversions, photohydrolysis reactions, photocleavage or photoextrusion reactions, photobleaching, etc.
2. Electrochemical conversions (amperometric or coulometric), oxidative or reductive reactions to convert an electrochemically inactive analyte into an electrochemically active derivative.
3. Enzymatic conversions, enzyme–substrate reaction detection, used to detect enzymes post-column via their reaction with substrate and formation of the conversion product, which may then be UV, FL, EC and/or MS active. This is detection of enzymes first separated by LC.
4. Microwave digestion reactions, post-column, used to digest proteins/peptides, nucleic acids and other biopolymers, leading to monomeric species that are more easily detected (EC) and/or derivatized before final detection (e.g. proteins → amino acids + OPA → tagged amino acids; UV, FL, or EC active).
5. Immunodetection (ID), post-column in LC, used to tag an antigenic species with a FL or enzyme-tagged antibody, leading to indirect detection of the untagged antigen via its complex formation in a sandwich format. A primary antibody must be immobilized on the solid ID support to initially capture the antigen after separation by LC.
6. Enzymatic conversion of a substrate, post-column, to form the turnover product with improved UV, FL or EC detection properties. This is detection of enzyme substrates, first separated by LC, then detected post-column by addition of enzyme in solution or via an immobilized enzyme reactor column, pre-detection.

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reagent to substrate, separation of derivative from sample components and reaction by-products, detector optimization for derivatives, chromatographic optimization of derivative peak shape, generation of standard derivative and structure confirmation, production of derivative calibration plot for quantitation, etc. The purity of the derivative peak in a sample must also be demonstrated by online photodiode array (PDA) or mass spectrometric (MS) methods. The derivatization reagent must be well characterized with regard to structure and its purity demonstrated. The reaction conditions need to be optimized to minimize reagent consumption, maximize derivative yield, and eliminate the formation or presence of reaction and reagent by-products that might interfere in the final separation and detection steps. It may even be necessary or desirable initially (pre-LC) to separate the excess reagent from the derivative and then introduce just the sample and the now-formed derivative into the analytical LC column.

Sometimes the reagents used have different detector properties from the final derivatives. The excess reagent at the end of the derivatization reaction may then be transparent under the optimized detection conditions for the derivative. It may even coelute together with the derivative peak, yet not be observed under such detection conditions. This reduces the need for initial separation of excess, unreacted reagent from the derivative and sample, and/or optimization of LC conditions so that the derivative peak appears completely resolved from all the other peaks.

Large versus Small Analyte Molecules and Their Derivatizations

It is generally easier to derivatize small molecules than large ones, since the rates of chemical reactions of very large biomolecules are usually orders of magnitude slower than those of smaller species. This is a function of effective chemical collisions, the number of chemical collisions per unit time between reactive sites, conformational preferences of large biomolecules, and the number of active sites available in a biomolecule. That is not to say that biomolecules cannot be successfully derivatized – they often are and can be – but the efficiency of derivatization (percent derivatization per unit time) versus smaller reactive species is usually much less. Also, the energy of activation needed to derivatize a primary amino group in a large molecule is often much larger than that for a very small molecule having the same functionality. This is, of course, a function of the neighbouring groups, conformational preferences, conformations available, hydrogen bonding within the biomolecule, and other factors. A considerable danger with derivatizing large molecules (typically biopolymers) stems from the fact that, in most cases, such a polymer possesses a number of reactive groups, for reasons just specified, which may differ in their reactivity. The result may be the formation of a number of products bearing the same tag in different mole per mole ratios. Although in enzymatic amplification techniques the formation of multiple products helps identification, in the situation just described the formation of multiple derivatization products should be avoided. The separation of such mixtures is often difficult, usually resulting in broad peaks and low plate counts. Moreover, it may be difficult to trace back which derivative was derived from which solute present in the original sample.

Numerous chemical reactions have been used to derivatize different classes of biomolecules in LC, usually with a high degree of success. However, the overall enhancement is always dependent on the particular tags used. That is, derivatization reactions that tag a specific site within the biomolecule sometimes lead to a single, and sometimes several, tags incorporated into the derivative. As a function of the tag, there

will be improved detector response, but perhaps much smaller chromatographic changes than with small molecules if the derivatization is carried out pre-column. Derivatizations are therefore often performed post-column. An ideal derivatization scheme would generate many derivatives from the original biomolecule, such as via enzyme amplification. This is already used to detect intact enzymes, but is used much less to detect proteins, peptides, nucleic acids, etc. Thus, the scheme described using post-column, microwave digestion of proteins, followed by a second post-column solution reaction with a FL derivatizing reagent (e.g. *o*-phthaldialdehyde, OPA), leads to many amino acids now detectable by FL methods. This is, perhaps, an ideal example of a general approach that greatly improves detectability of large molecules, such as via enzyme amplification for enzymes.

Offline versus Online Arrangements

It is also necessary to differentiate between offline and online arrangements (**Table 2**). In the offline mode the reactions occur away from the high performance liquid chromatography (HPLC) system, although there may be some examples that could be defined as either offline or online (e.g. in a sample vial in a carousel as part of an automated derivatization–injection system in LC). In the online mode the reaction chemistry occurs as part of the HPLC system, integrated into the instrumentation and analysis, and is time constrained and controlled. Thus there are four different and distinct types of derivatization approaches, or modes, for LC: (1) online, pre-column; (2) online, post-column; (3) offline, pre-column; and (4) offline, post-column (Table 2).

Pre-column versus Post-column Arrangements

The derivatization can be carried out in the pre-column or post-column mode, i.e. before or after the separation has taken place. In the post-column approach the derivatization reaction does not have to yield a single, stable product, provided that the reactions are reproducible. There are several serious disadvantages associated with this technique: (1) excess derivatization reagent must not interfere in the detection process; (2) reaction kinetics need to be rapid to allow real-time detection; (3) additional pumps are needed for a nonpulsating supply of derivatization reagent; (4) reaction solvents must be miscible with the separation mobile phase; and (5) an efficient mixing of derivatization reagent with the column effluent is required.

Table 2 Derivatization placement in LC

Mode	*Reaction sequence*
Pre-column, offline	Derivatization away from LC–injection–separation–detection
Pre-column, online	Derivatization on the LC–injection–separation–detection
Post-column, offline	Injection–separation–derivatization away from LC–detection
Post-column, online	Injection–separation–derivatization on the LC–detection

Pre-column derivatization is an alternative approach to post-column derivatization. One of its advantages is that derivatization is independent of the mobile phase and the reaction kinetics are not limited. Apart from an increase in detectability, pre-column derivatization can also improve the selectivity and chromatographic resolution of the overall method. Excess reagent present in the reaction mixture must be chromatographically resolved from the analyte derivative peaks, and/or be physically or chemically removed from the sample solution prior to injection. If several analytes yield the same derivative(s), then these would not be separable, and it would be impossible to determine which analyte was originally present in the sample. For example, the use of a substrate that could react with several enzymes, pre-column, would then lead to exactly the same product(s), preventing absolute identification of the enzyme actually present in the sample reaction mixture. More derivatizations have been performed online, post-column, as opposed to online, pre-column, or even offline, pre-column, for the above reasons, at least in LC areas. It is also possible to perform derivatizations *in situ*, or within the mobile phase. In this case derivatization reagent is placed in the solvents used for the LC separation. After separation has occurred, the eluent from the LC column can be heated to cause the reaction to occur, prior to the final detection stage. Unlike the online, post-column mode of operation, this does not require the addition of a mixing tee, heating coil, reagent pump, or ancillary tubing after the LC column.

Offline, Pre-column Derivatization

Offline, pre-column derivatizations have no extracolumn loss of efficiency and no solvent or kinetic limitations. Derivatization can be conducted under flexible reaction conditions or with harsh reagents. Offline derivatization can be optimized for high reaction yields and minimum by-products. Derivatization solvents need to be miscible with the chromatographic mobile phase. Otherwise, the derivatization solvents have to be evaporated and the residue of derivatives redissolved in a solvent compatible with the mobile phase. Offline derivatization does not need to give 100% theoretical yields, as long as there is good sample-to-sample reproducibility. However,

nonautomated offline pre-column derivatizations require operator attendance and manual manipulations.

Online, Pre-column Derivatization

Online, pre-column derivatization is accomplished by incorporation of a derivatization reagent into the flow scheme of the LC. Since all of the derivatized products are injected into the LC, this mode of derivatization does not have the solvent dilution problem observed in offline derivatization. There are several requirements for the conduction of online pre-column derivatization: (1) good chemical and/or pressure stability of derivatization reagents in organic solvent; (2) good solubility of derivatized products in the mobile phase; (3) no precipitate or gas generated in the derivatization; (4) compatibility of derivatization solvent with mobile phase; and (5) minimum volume of derivatization solvent or well packed solid-phase derivatization column. In online pre-column derivatization, the extraction and clean-up of complex samples often becomes part of the chromatographic operation, which can be automatically (computer/microprocessor interface) performed via switching of valves. Preliminary sample handling is minimized and automated derivatization procedures tend to provide better reproducibility.

Offline, Post-column Derivatization

This is perhaps the most unwieldy derivatization approach of all (Table 2). It involves separating the analyte of interest from the LC eluent prior to detection, performing a solution or solid-phase derivatization away from the instrumentation, manually or automatically, and then detecting the final derivatized solution. Automation is difficult, reproducibility is less than ideal, and even accuracy and precision falter, at times, because of a lack of total automatability. Thus, this mode receives the least emphasis in the literature and the lowest recommendation of application.

Online, Post-column Derivatization

In this approach (Table 2) injection–separation steps are followed by online derivatization, using automated, fully online instrumentation and methods. This technique utilizes post-column reactors (low dead volume mixing tees, knitted open-tubular reactors, low dead volume reaction coils, etc.) in which the chemical regents are introduced to the LC eluent. A delay time is needed (reaction dependent) to convert the analyte to product(s), and the entire solution, along with excess reagent(s), is introduced into the detector. This approach also allows for online liquid–liquid extraction, ion suppression (dual column ion chromatography), pH adjustment, organic solvent addition, basic hydrolysis reactions, additional chemical reactions modifying the solutes prior to the derivatization step (e.g. oxidation of imidazole ring in proline and hydroxyproline for their assay by the OPA reaction), enzyme addition, and the use of post-column, immobilized reagents or enzymes. There are many chemical reactions that have been employed post-column online: sequential reactions, solid-phase/catalytic enhanced reactions (e.g. carbamate detection), microwave digestion of proteins, photochemical reactions, etc. However, there are severe constraints or requirements on the nature of the reagent solvent and solution that can be mixed with the LC effluent, detector transparency of such solvents, prevention of analyte derivative precipitation before detection, mixing of reagents with analyte, lack of mixing noise, need for additional instrumentation, mixing tees, connecting joints, and extra tubing connections. Nevertheless, at least in LC areas, this particular mode has been the most widely employed and applied.

Specific Recommendations for Successful Application of Derivatization in Liquid Chromatography

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It is clear that there are numerous approaches to successful derivatization possible in various modes of LC, including reversed-phase, ion exchange, normal phase and hydrophobic interaction. There are perhaps too many choices as to which specific reagent will prove applicable for a new analyte, or how to best optimize and apply any given reagent, much less what might prove the optimal LC conditions for the final derivatives. A rational approach to derivatization for all LC is called for. Such rational designs for method development, optimization and validation in HPLC are available from the literature. A rational approach to developing, optimizing and then validating a derivatization method for LC is described below.

1. Know the structure of the analyte(s), what functional groups are present for tagging, and what types of reactions might be employed. A good knowledge of organic chemistry is needed and available at this stage. Some of the existing texts on derivatizations for HPLC should be utilized.
2. What are the requirements of the final derivatization-LC method? It is necessary to decide what detection limits are needed, what sample matrices will be analysed, what limits of quantitation must be realized, what resolution (sample dependent) will be needed, and so forth.

3. What is known in the literature about the LC of the analyte of interest, as a standard pure compound (without regard to sample matrices yet)? Are conditions reported for underivatized analysis, and what conditions have been already described and optimized? Could these be eventually utilized for simple derivatives of the original analyte? What modifications might be needed to resolve the analyte derivatives? Are any tagging methods already reported for GC or thin-layer chromatography (TLC) that might prove applicable in LC? What types of reagents have been described? What were the specific reaction conditions already optimized for this derivatization scheme?
4. Perform simple, test tube reactions on a standard of the analyte offline, away from the LC instrument, to optimize reaction conditions and to demonstrate the nature of the products formed, their number, derivatization yield, ease of product work-up prior to LC, etc. Utilize TLC, gas chromatography (GC), LC, and whatever other analytical tools are available to determine which reagents will tag the analyte, the nature of the products formed. Follow the optimization steps described below.
5. Optimize the derivatization conditions in terms of the usual reaction parameters: time, solvent, pH, temperature, catalysts. This can be performed univariately or multivariately, even using computer algorithms (simplex/multiple routines) to realize surface maps of conditions leading to optimal formation of the desired derivative. Whatever the optimization routine used, the final conditions need to be compatible with pre- or post-column LC reaction requirements (instrumental, solvents, mixing). Optimize reaction conditions and demonstrate formation of the desired derivative *before* introduction into the LC instrument.
6. Demonstrate the formation of derivative, nature of the derivative (structure), purity of standard derivative, per cent derivatization (yield), etc., using standard organic chemistry methods (elemental analysis, mass spectrometry, Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy). What is the nature of the derivative obtained from the analyte? What is its exact structure, solubility, stability to various LC solvents, detection properties (UV, FL, EC), etc.?
7. How does the final derivatization approach change the possible ionization states of the original analyte? What modifications to the separation conditions of the original, untagged analyte must now be made to accommodate the nature of the derivatized species (e.g. ion exchange chromatography (IEC) changes)? Will the new tag(s) induce additional charges on the original analyte that will then affect LC mobility, migration times, resolutions, etc.? Will the tags induce unwanted hydrophobic properties to the tagged species affecting solubility, migration tendencies, resolution, efficiency and peak shape? How do we then accommodate such structural and physical/chemical property changes, how do we know what those changes really are before any LC methods development is pursued? Will the newly tagged species still permit host–guest complexation, such as with cyclodextrins, crown ethers and other complexation additives to the LC buffer?
8. Now utilize the standard derivative to optimize the LC conditions, again consulting the literature to determine if this derivative or an analogous structure has already been described along with specific LC operating conditions. Utilize those conditions or slight variations to realize optimized LC conditions for your standard derivative. This may require optimization by univariate or multivariate methods, perhaps using computer algorithms, varying one parameter at a time to generate a surface map demonstrating optimized conditions. This is similar to resolution maps in LC via DryLab from LC Resources. There are other computer programs in the literature that might prove useful in this area of LC separation optimization for the standard derivative.
9. Demonstrate analytical figures of merit with standard derivative, based in part on original method/assay requirements, such as linearity of calibration plots possible (UV, FL, EC), detection limits, limit of quantitation, accuracy and precision of quantitations possible, robustness of the LC conditions to small operational changes (pH, temperature, solvent, ionic strength, voltage applied, sample introduction, etc.), time per analysis, cost per analysis, instrument/method preparation, etc. This is still all derived for standards of the derivative, and not yet with actual analyte or samples.
10. Demonstrate analytical figures of merit with standard analyte, exactly as above, but now introducing the actual derivatization steps required to convert the original standard analyte into the derivative.
11. Demonstrate analytical figures of merit with actual sample containing *known* levels of analyte, including all method requirements: limit of quan-

titation (LOQ), limit of detection (LOD), linearity of calibration plots, ruggedness, robustness, reproducibility, repeatability, accuracy/precision of quantitations, time per analysis, cost per analysis and sample preparation requirements.

12. Validate final, optimized method with known samples containing known levels of analyte using double-blind spiking, standard reference materials (if available), comparison with currently accepted method on split, spiked samples (known levels), and finally interlaboratory collaborative studies. Assemble all final data in terms of accuracy and precision, reproducibility from laboratory to laboratory, repeatability within one laboratory, ruggedness from laboratory to laboratory, robustness within any given laboratory, all in terms of qualitative identification of analyte present, and then final quantitative information in terms of accuracy and precision of such measurements.
13. Write up final procedure and protocols for performing the final, overall derivatization-LC method, including the necessary sample preparation steps, isolation of analyte from matrix (if required) before derivatization, possible derivatization of analyte in sample matrix followed by isolation of derivative, or derivatization of analyte in sample matrix with direct injection of crude mixture into LC with minimal (if any) sample preparation (dilute/shoot). Include all possible procedures and reagents, chemicals, solvents and instrumentation needed for another laboratory to reproduce, repeat, and obtain valid results using the newer method in their hands/laboratories.
14. Distribute the final protocols and procedures to all those laboratories that participated in the interlaboratory collaborative studies, so that they can validate and demonstrate reproducibility of the overall optimized methods involving derivatization-LC operations and conditions.

Problems and Pitfalls in Using Derivatization in Liquid Chromatography

There are some potential problems and pitfalls in the routine use of derivatizations in LC. Major amongst these is the need to remove the excess reagent and/or its hydrolysis and thermal degradation products from the final derivatization solution prior to detection. This can be accomplished by an initial sample clean-up offline, and/or by addition of a large amount of another reactive compound to consume all of the excess reagent to form a single known derivative easily separated from the analyte's derivative. Sometimes the LC conditions themselves may resolve the excess reagent and any of its hydrolysis/by-products from the desired derivative. Other approaches utilize a derivatizing reagent that, together with its hydrolysis/by-products, does not appear in the final chromatogram because it has very different detector properties from those of the analyte's derivative.

Another possible problem in utilizing derivatization involves a low per cent conversion to the desired derivative. This can be improved by forcing the reaction conditions, working at elevated temperatures for longer periods of time, invoking a suitable catalyst and by increasing the concentrations of analyte and reagent. Sometimes isolating the analyte from the sample on a solid support, followed by reaction with the usual derivatization solution, can lead to a much faster and more efficient reaction and conversion. In general the higher the per cent conversion, the easier it is to detect trace levels of analyte in complex matrices, such as biofluids.

Another problematic area has to do with reactions from other components in the sample mixture, besides that of the desired analyte, leading to a complex mixture of derivatives difficult to resolve by LC and/or detection methods. This can be improved by selectively isolating the analyte of interest from the sample matrix prior to derivatization, followed by the desired reaction conditions and introduction of the derivative into the LC system. This can more easily be accomplished by combining affinity LC with another LC mode, such as reversed-phase, so that the affinity step isolates the analyte of interest. This is then followed by a derivatization on the affinity support with the analyte immobilized, or initial elution of the analyte from this support, solution reaction, and then introduction into the second LC system. A simple, solid-phase affinity extraction column can be used to isolate the desired analyte from the complex sample, and prepare it for the desired, homogeneous (solution) or heterogeneous (solid-phase) derivatization reaction.

Yet another possible pitfall has to do with the formation of several derivatives from the analyte, rather than the usual (desired) production of a single, homogeneously tagged derivative. It is usually desired to form a single, homogeneous derivative with good chromatographic and detector properties. However, if there are several possible reactive sites on the analyte, then it is always possible that more than one product will result. This can be avoided by using reaction conditions that force all sites to be tagged,

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leading to a single product, or by preventing some of the sites from reacting by using suitable reaction conditions or protecting groups that will then leave only a single site left to react. In the case of protein or biopolymer derivatizations, multiple products are usually formed, leading to several LC peaks that then raise detection limits and make identification of the original protein and quantitation more difficult, especially at trace levels. In general, homogeneous (uniform) tagging of biopolymers is always problematic, though conditions are currently being developed that may eliminate such difficulties.

It is possible that the reaction conditions required for derivatization may cause the analyte itself to degrade, even as it reacts with the reagents. The degradation products can also react with the very same tagging reagent. This leads to a multiplicity of products, rather than a single homogeneous derivative, again making quantitation at trace levels and identification of the original analyte more difficult. However, this complex mixture of products can be forced to elute as a single, sharp peak by using suitable LC conditions. This can then function as a suitable peak for quantitation and identification of the analyte of interest.

Finally, there are the issues of reagent stability, purity, uniformity and shelf-life, all important areas when using a reagent over a long period of time for numerous analyses. Conditions must be found that provide a pure reagent with good shelf-life, long-term stability during the course of the reaction and storage, available from several commercial vendors at reasonable cost and amounts, and available in high purity and consistency. In most cases, such commercial reagents are indeed available for many LC applications today.

Conclusions and Summary

This article has provided an overview of derivatization for LC. It is clear that this approach has undergone significant developments over the past few decades, to the point where it is now a mature area of LC science. Numerous books and reviews have appeared in recent years, and the literature continues to grow. Several excellent primers are available on the use of derivatization in LC and other separation areas, such as capillary electrophoresis (CE). Derivatization serves several useful functions in LC, but can especially improve chromatographic performance and peak shape for the original analyte, improve detector response to permit trace determinations, and improve quantitation for the original analyte by improving signal-to-noise ratios in complex sample matrices.

Derivatization can also stabilize an otherwise reactive analyte by the formation of a more stable derivative. The formation of multiple derivatives, using either solution or solid-phase (mixed-bed) approaches, has enabled improved qualitative identification of an analyte, as well as confirmation of quantitation by providing two to three different peaks for such purposes, all from the same sample undergoing one or a series of tagging reactions. Automation of derivatization, both pre- and post-column and online or offline, has developed such that it has become virtually a routine part of LC analysis. It is quite common to perform derivatization of amino acids pre-column, offline or online, in order to improve the identification and quantitation of these species, for example in a protein hydrolysate or intravenous solution. Derivatization for trace level detection of many analytes has also become commonplace, particularly when combined with preconcentration as part of the sample preparation–derivatization–LC steps. These tagging approaches permit the trace analysis of many analytes in complex sample matrices that otherwise would not be detectable by direct LC analysis.

Derivatization has thus become very commonplace in much of LC analytical work and applications. A wide variety of suitable reagents are commercially available, providing enhanced detection in several modes (UV, FL, EC and MS). Derivatization approaches are being developed for proteins and peptides that would lead to directed fragmentation and/or improved ionization for lowered detection limits in various forms of mass spectrometry or LC-MS. These efforts to develop improved derivatization reagents for further LC-detector applications will undoubtedly continue for many years to come.

See also: **II/Chromatography: Liquid:** Detectors: Ultraviolet and Visible Detection. **III/Peptides and Proteins:** Liquid Chromatography.

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Detectors: Electron Spin Resonance

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Introduction

The role of radicals in (bio-)chemical reactions is currently becoming increasingly significant. Free radicals may be generated by any kind of irradiation and contribute essentially to many aging processes in many materials, particularly in the presence of oxygen. They are even able to cause manifold organic damages as in lipid peroxidation or in inflammatory diseases. Organic reperfusion injuries after ischaemia are currently the subject of intensive research activities. On the other hand, stable free radicals are used practically in a number of applications in many fields, e.g. as additives in industrial processes such as polymerization or as analytical tools in research on membrane, emulsion and surface properties of materials or formulations. The utilization of such substances as protective additives, e.g. for process control or as research tools, has stimulated interest in the synthesis of new compounds of this class. The increasing search for radicals is paralleled with a rising demand for methods to detect, identify and quantify them. In the context of separation techniques, this means having a technique at hand to trace them in eluted fractions.

High chemical reactivity combined with low specificity is typical of the majority of radicals. As a consequence, solutions containing such substances are likely to alter their composition within a short time owing to the decreasing content of reactive components and to the accumulation of reaction products. This kind of change can easily be monitored by chromatographic methods. Having separated all the constituents at a given time, it may become necessary to identify original or intermediate radicals to evaluate the particular stage of an ongoing reactive process. However, the detectors routinely used in HPLC cannot indicate directly any radical present in the separated fractions. The most advanced method suitable for this purpose is electron spin resonance spectroscopy (ESR), but most ESR spectrometers are currently installed as large and heavy instruments, not at all suitable as detectors for chromatographic methods. It will be shown here that this kind of spectroscopic method can be realized with devices of table-top size that can easily be integrated into any chromatographic separation line.

Electron spin resonance (or electron paramagnetic resonance, EPR) spectroscopy is the only direct method to measure radicals since it is based on the existence of unpaired electrons. Likewise, paramagnetic metal complexes are also sensitive to this spectroscopic method. A substantial advantage for the study of radical reactions would be the rapid analysis of a fraction directly upon separation to avoid changes caused by putative consecutive reactions. This can only be achievable by direct coupling of the separator (HPLC) with the specific detector (ESR) in the shortest possible way without any unnecessary dead volume (long tubing lines or valves). An absolute prerequisite for such an instrumental set-up is a spectrometer of a size that allows installation at the site of the sample separation, and not necessarily vice versa. The other problem is synchronization of sample separation and recording of a spectrum which requires a definite period of time. Both problems have been solved in the on-line coupling of HPLC and ESR spectroscopy described here.

ESR Spectroscopy as Detector

An introduction into the principles of ESR (or EPR) spectroscopy can be found in most textbooks on physical chemistry or in specialized monographs. Since this spectroscopic technique is rather uncommon

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